TOXICOLOGY AND APPLIED PHARMACOLOGY 79, 69–82 (1985)

The Joint Neurotoxic Action of Inhaled Methyl Butyl Ketone Vapor and Dermally Applied *O*-Ethyl *O*-4-Nitrophenyl Phenylphosphonothioate in Hens: Potentiating Effect[1]

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Received August 23, 1984; accepted January 8, 1985

The Joint Neurotoxic Action of Inhaled Methyl Butyl Ketone Vapor and Dermally Applied *O*-Ethyl *O*-4-Nitrophenyl Phenylphosphonothioate in Hens: Potentiating Effect. ABOU-DONIA, M. B., LAPADULA, D. M., CAMPBELL, G., AND ABDO, K. M. *Toxicol. Appl. Pharmacol.* **79,** 69–82. The neurotoxic action of inhaled technical grade methyl butyl ketone and dermally applied (*O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate (EPN) was studied. Three groups of five hens each were treated 5 days/week for 90 days with a dermal dose of 1.0 mg/kg of EPN (85%) on the unprotected back of the neck. These groups were exposed simultaneously to 10, 50, or 100 ppm of technical methyl butyl ketone (MBK; methyl *n*-butyl ketone:methyl isobutyl ketone, 7:3) in inhalation chambers. A fourth group was treated only with the dose of EPN and a fifth group with only 100 ppm MBK. The control consisted of a group of five hens treated with a dose of 0.1 ml acetone. Treatment was followed by a 30-day observation period. Simultaneous exposure to EPN and MBK greatly enhanced the neurotoxicity produced when compared to the neurotoxicity produced by either chemical when applied alone. Continued exposure to EPN and MBK resulted in earlier onset and more severe signs of neurotoxicity than exposure to either individual compound. The severity and characteristics of histopathologic lesions in hens given the same daily dermal dose of EPN in combination with inhaled MBK depended on the MBK concentration. Histopathologic changes were more severe and prevalent in the 100 ppm MBK:1 mg/kg EPN group than in the others. In this group, Wallerian-type degeneration was seen along with paranodal axonal swellings. The morphology and distribution of these lesions were characteristic of those induced by MBK. In the 50 ppm MBK:1 mg/kg EPN group axonal swelling was evident but not clearly identifiable as paranodal. Hens treated with 10 ppm MBK:1 mg/kg EPN had minimal lesions with low incidence of axonal swellings. These were not as large as those seen in MBK neurotoxicity, but instead resembled the histopathologic lesions caused by EPN. The results indicate that the combined treatment gave a value for neurotoxicity coefficient which was two times the additive neurotoxic effect of each treatment alone. Pretreatment with three daily ip doses of 5 mmol/kg technical grade MBK or methyl *n*-butyl ketone (M*n*BK), equally increased chicken hepatic microsomal cytochrome *P*-450 content. Also, hepatic microsomes from MBK-treated hens metabolized [^{14}C]EPN *in vitro* to [^{14}C]EPN oxon to a much greater extent than those from control hens. These results suggest that MBK potentiates the neurotoxic effect of EPN, at least in part, by increasing the metabolic activation of EPN to the more neurotoxic metabolite EPN oxon. A similar mechanism may account for the potentiating effect of EPN on MBK neurotoxicity, by increasing the formation of the more neurotoxic agent 2,5-hexanedione.

The insecticide leptophos (*O*-4-bromo-2,5-dichlorophenyl) *O*-methyl phenylphosphonothioate) and *n*-hexane were implicated in a severe neurologic dysfunction among 12 workers at a Bayport, Texas plant (Staff Report, 1976; Xintaras *et al.*, 1978). Leptophos can produce delayed neurotoxicity (OPIDN) in test animals, e.g., the adult chicken

[1] A preliminary account of this work has been presented (Abou-Donia, 1983).

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0041-008X/85 $3.00

(Abou-Donia *et al.*, 1974; Abou-Donia and Preissig 1976a,b). Although leptophos has been banned, other delayed neurotoxic pesticides are produced in factories where simultaneous exposure to hexacarbon solvents is possible.

Both *n*-hexane and leptophos belong to classes of chemicals that produce central–peripheral distal axonopathy (Spencer and Schaumburg, 1980). Neurotoxic aliphatic hydrocarbons include *n*-hexane and the related chemicals methyl *n*-butyl ketone (M*n*BK), 2,5-hexanediol (2,5-HDOH), and 2,5-hexanedione (2,5-HD). Some organophosphorus compounds such as tri-*o*-cresyl phosphate (TOCP) produce delayed neurotoxicity (Smith *et al.*, 1930; Abou-Donia, 1981). This effect of TOCP was first observed in humans; it was later demonstrated in some animal species (cats, dogs, cows, mallard ducklings, and chickens) but not in others (rodents and some nonhuman primates). Although neuronal lesions produced by either class of chemicals are characterized by Wallerian-type degeneration of axons and myelin in the central and peripheral nervous systems, these histopathologic changes differ in both the morphology and distribution of the lesion (Abdo *et al.*, 1982; Abou-Donia *et al.*, 1982). These results are explained by the dissimilarities between the two classes of chemicals, which suggest that they produce their respective neurotoxicities via different mechanisms (Abou-Donia, 1983).

The joint action of neurotoxic chemicals may result from (1) modification of a chemical or physical characteristic of the molecules, leading to changes in their biological action, (2) interactions at the neurotoxicity target, (3) indirect interactions through modification of xenobiotic metabolizing enzymes, or (4) interaction at the absorption site, thus changing the body burden of the neurotoxic chemical. Synergism or potentiation occurs when the neurotoxic action produced by applying two or more chemicals is much greater than what would be expected from the simple summation of the effects induced by each chemical (Hodgson, 1980). Synergism takes place when one component has little or no intrinsic neurotoxicity. On the other hand, potentiation occurs when both components have intrinsic neurotoxicity (Rentz, 1932).

The present study was designed to investigate the neurotoxic action of concurrent exposure to the technical grade of the neurotoxicants EPN (*O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate) and methyl butyl ketone (MBK; methyl-*n*-butyl ketone:methyl isobutyl ketone, 7:3). EPN was applied dermally and MBK via inhalation, the most likely routes of workers' exposure to these chemicals. The test animal was the adult chicken, since it is sensitive to neurotoxicity produced by both chemicals (Abou-Donia, 1981; Abdo *et al.*, 1982; Abou-Donia *et al.*, 1982). Specifically, three aspects were examined: (1) the time course of neurotoxicity signs, (2) the extent of the neurologic deficit, and (3) severity and frequency of histopathologic changes. Also, the mechanism of this potentiation was investigated by determining hepatic microsomal cytochrome *P*-450 and the *in vitro* metabolism of [^{14}C]EPN by microsomes.

METHODS

Chemicals

Radioactive, analytical, and technical grade (85%) EPN *O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate and EPN oxon (*O*-ethyl *O*-4-nitrophenyl phenylphosphonate) were provided by E. I. DuPont de Nemours Company, Inc., Wilmington, Delaware. [U-*phenyl*-^{14}C]EPN (*O*-ethyl *O*-4-nitrophenyl [^{14}C]phenylphosphonothioate) had a specific activity of 1.65 μCi/mmol. Technical grade methyl butyl ketone containing 70% methyl *n*-butyl ketone (2-hexanone, M*n*BK) and 30% methyl isobutyl ketone (4-methyl-2-pentanone, M*i*BK) was obtained from Eastman–Kodak Company, Kingsport, Tennessee.

Animals

Leghorn laying hens, 14 months old, weighing 1.59 $\pm$ 0.07 kg ($\bar{x}$ $\pm$ SE) and specified-pathogen free were used (Featherdown Farm, Raleigh, N.C.). Groups of five

hens, randomly assigned, were housed in single-tier, stainless-steel inhalation chambers in a humidity (40 to 60%)- and temperature (21 to 23°C)-controlled room with a 12-hr light cycle before and during the experiment. The chickens were allowed to adjust to their environment for a week before treatment. They were supplied with feed (Layena chicken feed, Ralston–Purina Co., St. Louis, Mo.) and water *ad libitum*.

Treatment of Birds

Joint neurotoxicity of EPN and MBK. A daily dose of 1.0 mg/kg EPN 85% in 0.1 ml of acetone was applied, 5 days a week for 90 days, to the unprotected back of the neck of four groups of hens (five hens each). Three of these groups were exposed simultaneously to 10, 50, or 100 ppm MBK in inhalation chambers. Of two other groups (five hens each), one was similarly exposed to 100 ppm MBK, while the other received a daily dermal dose of 0.1 ml acetone. Surviving birds were kept out of the chambers for a 30-day observation period. Body weights were monitored weekly, and hens were examined daily for neurologic deficits. The dose regimens of EPN and MBK alone were selected because each produced a threshold neurotoxic effect when used alone.

Effect of microsomal enzyme inducers. Groups of four hens were treated with a daily ip dose, for 3 consecutive days, of 5 mmol/kg M*n*BK or MBK in 5 ml corn oil. Controls consisted of four hens similarly treated with corn oil. All hens were anesthetized by carbon dioxide and killed by decapitation 24 hr after the last dose.

Inhalation exposure to MBK vapor. The chickens were exposed to MBK vapor in portable-type stainless-steel animal inhalation chambers (Young and Bertke Co., Cincinnati, Ohio) described previously (Abdo *et al.*, 1982). M*n*BK and M*i*BK vapor mixture was generated in a 1-liter three-necked flask half filled with technical grade MBK. A 500-ml separatory funnel that served as a reservoir of MBK was connected to one neck, a tank of compressed air was attached to another, and the third to a 400-ml condenser. Teflon tubing connected the condenser to the chamber. Both the Teflon tubing and the condenser were wrapped with heating tape that was heated to 50°C to prevent condensation of MBK mixture vapor. Airflow from the tank swept the vapor into the chamber. The desired concentration of MBK vapor was maintained by adjusting the airflow from the air tank.

Gas Chromatographic Analysis of MnBK and MiBK

A Gow-Mac gas chromatograph (Series 750, Gow-Mac Instruments, Bound Brook, N.J.) equipped with a 6 ft. × 2 mm o.d. coiled glass column packed with Tenax GC 60/80 mesh, a flame ionization detector, and a chart recorder were used in monitoring M*n*BK and M*i*BK concentrations in the inhalation chambers. Teflon tubing, used to draw air samples from the center of the cage, was connected to a 2-mm injection loop attached to a valve for injecting the air into the gas chromatograph. The column was conditioned at 250°C with purified He at 70 ml/min for 24 hr. Temperatures were 150°C for column and 160°C for both detector and injector block. Gas flow rates (cm^3/min) were the following: He (carrier gas), 30; H_2, 20; air, 300. Standard curves for M*n*BK and M*i*BK were obtained by plotting concentration against peak areas for each chemical as described previously (Abdo *et al.*, 1982).

Clinical Evaluation

Control and treated hens were observed daily inside the exposure chamber and moving freely outside the chamber. Clinical signs of neurotoxicity were categorized into four stages: mild ataxia (T_1), in which there was a reluctance to walk and diminished leg movement; gross ataxia (T_2), recognized by change in gait and disturbance of leg movement; severe ataxia (T_3), characterized by unsteadiness and occasional falling on the floor; ataxia with near paralysis (T_4), marked by inability to walk. The most severe condition is paralysis in which hens were unable to stand. For graphical presentation, hens were assigned numerical values of 1 to 4, corresponding to T_1 to T_4 stages of ataxia, and normal animals the value of zero (Abou-Donia, 1978).

Histopathologic Studies

Nervous tissues were excised shortly after death from hens that died and from those killed at the end of the experiment. Hens whose clinical condition deteriorated and became moribund were killed before the end of the experimental period. Anesthetization of hens with CO_2 was followed by opening of the thorax and perfusing 10% neutral phosphate-buffered Formalin through a cannula into the ascending aorta via the left ventricle with the aid of a pump (Manostat varistalic pump, Fisher Scientific, Raleigh, N.C.). Sciatic, tibial, and peroneal nerves, and their branches to the ankle, along with the spinal cord, were fixed for at least a week in a buffered-Formalin solution (Abou-Donia and Pressig, 1976a). Cross sections and parasagittal longitudinal sections near the midline were prepared from the cervical, thoracic, and lumbar regions of the spinal cord. Peripheral nerves were prepared in cross and longitudinal sections. Tissues were dehydrated in graded ethanol and imbedded in paraffin or glycol methacrylate. Paraffin sections (8 μm) from the spinal cord were stained with hematoxylin and eosin (H&E) combined with luxol fast blue (LFB) or Glees stain. Sections from peripheral nerves also were stained with Holmes' stain. Glycol methacrylate sections of the spinal cord and peripheral nerves (1 to 2 μm) were stained with toluidine blue.

Enzymatic Analysis

The livers were quickly removed after decapitation. Hepatic microsomes prepared as described previously (Schenkman and Cinti, 1978) were washed, frozen with liquid nitrogen, and stored at −70°C. Microsomal suspensions containing 2 to 5 mg of protein per milliliter were prepared. Cytochrome *P*-450 was determined by the Omura and Sato (1964) method and expressed as nanomoles of cytochrome *P*-450 per milligram of protein. Microsomal proteins were determined by the Bradford (1976) method using bovine plasma γ-globulin (Bio-Rad Laboratories, Richmond, Calif.) as standard.

In Vitro Metabolism of EPN with Hepatic Microsomes

Incubation of EPN in vitro. Metabolism of EPN was carried out using hepatic microsomes from hens treated, for 3 consecutive days, with 5 mmol/kg MBK in corn oil and from control hens given corn oil alone as described above. Microsomes equivalent to 1 mg protein were incubated in 0.5 ml of 10 mM Tris–HCl buffer, pH 7.4, containing 150 mM KCl, 5 μmol of NADPH, and 1 μmol [^{14}C]EPN in 10 μl acetone.

Extraction procedure. After an hour incubation period, the reactions were terminated by the addition of 0.5 ml ice-cold acetone to precipitate proteins which were then centrifuged. The resultant supernatant fraction was concentrated to dryness with nitrogen. Residues were dissolved in 200 μl acetone for thin-layer chromatographic (TLC) analysis.

Thin-layer chromatography. Gelman-type SA, ITLC silicic acid-impregnated glass-fiber sheets (Gelman, Ann Arbor, Mich.) were used (Abou-Donia and Ashry, 1978). A mixture of EPN and its metabolic product (EPN oxon) was added to the solvent extracts of the [^{14}C]EPN reaction mixtures. The chromatographic separation of standards and the labeled unknown was carried out on the ITLC sheets using the solvent system *n*-hexane: benzene:acetic acid (5:5:1). The standards were detected by their color in iodine vapor. Next, the TLC sheets were cut into 5-mm strips, placed into scintillation vials, vigorously mixed with the scintillation medium (toluene: ethylene glycol monomethyl ether (4:1 v/v) containing 5 g of 2,5-diphenyloxazole (PPO) and 0.2 g of 1,4-bis[2-(phenyloxazolyl)benzene] (POPOP)/liter). EPN and its metabolites were quantified by counting radioactivity in a scintillation spectrometer (Packard Instrument Co.) at 5°C. Counts were corrected for dilution, quenching, background, and counting efficiency.

Determination of Neurotoxicity

Neurotoxicity index. Quantification of neurotoxicity was carried out by calculating the neurotoxicity index (NTI) for each treatment (Abou-Donia *et al.*, 1982). The NTI was determined by ranking the hens according to (1) the time of onset of neurologic deficit, (2) the severity of neurologic dysfunction, and (3) the severity and frequency of histopathologic alterations (Jonckheere, 1954). Ranking assignment started with minimal changes in the three areas. The NTI for a treatment was determined as the mean of the three ranks of hens in each of the three categories.

Coneurotoxicity coefficient. The joint neurotoxic action produced by applying two or more neurotoxic agents was determined by calculating the coneurotoxicity coefficient (CNC) according to the equation

$$\text{CNC} = \frac{\text{experimentally determined NTI for chemicals } 1, 2, \ldots, n}{\text{NTI}_1 + \text{NTI}_2 + \cdots + \text{NTI}_n}.$$

Synergism or potentiation occurs when the CNC is greater than one, antagonism, when the CNC is less than one, while CNC equaling one indicates an additive effect.

The joint neurotoxic action produced by treating hens with EPN and MBK simultaneously was determined by calculating $\text{CNC}_{\text{EPN,MBK}}$ as

$$\text{CNC}_{\text{EPN,MBK}} = \frac{\text{experimentally determined NTI}_{\text{EPN,MBK (experimental)}}}{\text{NTI}_{\text{EPN}} + \text{NTI}_{\text{MBK (Expected)}}}$$

where NTIs are as defined above.

Statistics

Significance of the differences between the weight and enzymatic measurements of control and treated hens was assessed by a two-tailed Student's t test. A p value of 0.05 or less was considered significant.

RESULTS

Analysis of MnBK, MiBK, and Technical Grade MBK

Using gas chromatography, the retention times for M*n*BK and M*i*BK were 2.5 and 1.8 min, respectively. The mean M*n*BK concentration in the inhalation chambers was 70 ± 5.2%. The concentration of MBK vapor mixture in the chamber varied less than 10% from the target concentration.

Body Weight Changes

Hens treated with a daily dermal 1.0 mg/kg EPN for 5 days a week lost little weight

at onset of ataxia and most of the weight loss was regained by termination (Table 1). Exposure to 100 ppm MBK 5 days a week caused an insignificant increase in body weight. Simultaneous exposure of hens to a dermal dose of 1.0 mg/kg EPN and to a vapor exposure of 10, 50, or 100 ppm MBK caused a significant body weight loss at onset of ataxia. The weight loss persisted during the exposure period; however, these chickens regained most of the lost weight by the end of the experiment. The weight of acetone-control hens (0.1 ml/kg/day) did not change during the study.

Cholinergic and Narcotizing Effects

Daily exposure to a dermal dose of 1.0 mg/kg EPN, or a vapor exposure of 100 ppm MBK, or a combination of the two compounds, 5 days a week did not produce signs of the cholinergic effect of EPN or the narcotizing effect of MBK.

Functional Disturbances Caused by Neurotoxicity

Daily dermal application of 1.0 mg/kg EPN for 5 days a week caused mild ataxia (T_1), which later became severe (T_3)(Fig. 1). After the daily treatment of EPN had stopped, the clinical condition of the severely ataxic (T_3) hens regressed to gross ataxia (T_2). Inhalation of 100 ppm MBK vapor 5 days a week produced mild ataxia (T_1) after 39 days, a condition that did not change throughout the study.

The severity of the neurologic deficit resulting from treatment with a daily dermal dose of 1.0 mg/kg EPN and a daily MBK vapor exposure depended on the MBK concentration. Thus, concurrent exposure of hens to 1.0 mg/kg EPN and 10 ppm MBK vapor produced gross ataxia (T_2); when combined with a 50-ppm MBK vapor exposure, severe ataxia (T_3) developed. Joint treatment with 100 ppm MBK vapor induced near paralysis stage (T_4). The clinical condition of these hens remained unchanged during the observation period.

Histopathologic Alterations

Nervous tissue from hens exposed continually to 100 ppm MBK or a daily dermal dose of 1 mg/kg EPN 5 days a week for 90 days did not exhibit histopathologic changes

TABLE 1

CHANGE IN WEIGHT OF CONTROL AND HENS SIMULTANEOUSLY EXPOSED TO SUBCHRONIC (90 DAYS) INHALATION OF TECHNICAL GRADE METHYL BUTYL KETONE AND DERMAL APPLICATION OF EPN[a]

EPN (mg/kg)	MBK (ppm)	Initial weight (kg)	% Initial weight at		
			Onset of ataxia	End of exposure	Termination
1.0	0	1.68 ± 0.06	93.4 ± 4.6	93.1 ± 3.6	99.0 ± 2.3
0	100	1.50 ± 0.08	105.9 ± 7.2	101.1 ± 6.1	103.5 ± 6.7
1.0	10	1.54 ± 0.05	72.1 ± 1.9[c]	75.2 ± 5.0[b]	93.0 ± 2.6
1.0	50	1.55 ± 0.0	82.5 ± 4.5[c]	88.6 ± 3.8[c]	98.6 ± 4.0
1.0	100	1.47 ± 0.07	86.5 ± 1.7[c]	89.6 ± 2.1[c]	104.0 ± 0.5
0	0[d]	1.72 ± 0.09			101.5 ± 2.5

[a] Groups of five hens were exposed to a daily dermal dose of EPN (85%) in acetone, MBK (70% M*n*BK and 30% M*i*BK) vapor, or both simultaneously in inhalation chambers, 5 days a week for 90 days. Surviving birds were kept out of the chambers for a 30-day observation period.

[b] Significant difference $p < 0.02$.

[c] Significant difference $p < 0.05$.

[d] Control hens were treated with a daily dermal dose of 0.1 ml acetone for 90 days, then kept for a 30-day observation period.

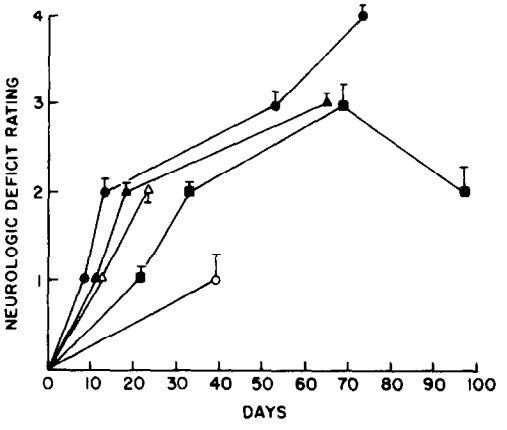

FIG. 1. Neurologic deficits in hens exposed to 100 ppm MBK (○), 1 mg/kg daily dermal dose of EPN (■) and simultaneous exposure to 1 mg/kg EPN and 10 (△), 50 (▲), or 100 (●) ppm MBK. Neurologic deficit rating is described under Methods. The results show days before onset of each stage of neurologic deficit and represent the mean from three hens ± SD.

(Table 2). On the other hand, simultaneous exposure to a 1-mg/kg dermal dose of EPN and 10, 50, or 100 ppm MBK vapor produced lesions in some hens. Concurrent exposure to 10, 50, or 100 ppm MBK vapors produced changes of the spinal cord in one, three, and four hens, respectively. Also, two hens from the first two groups and one from the third group exhibited changes characterized by occasional swollen axons but unaccompanied by fragmentation, phagocytosis, or myelin loss. Only one hen exposed to 1 mg/kg dermal EPN and 100 ppm MBK presented these lesions in peripheral nerves (Fig. 2).

The results indicate that the character of the histopathologic lesions in hens treated simultaneously with a daily dermal dose of 1 mg/kg EPN and various concentrations of MBK depended on the concentration of inhaled MBK (Table 2). When 100 ppm MBK was used, the lesion type seen in the nervous system of exposed hens was characteristic of MBK. Thus, axonal swellings were giant and paranodal in the spinal cord and peripheral nerves (Figs. 3 and 4). Axonal swellings were also intensely stained with eosin, indicating accumulation of neurofilaments. Swollen axons were common in the ventral columns. Degenerated axons were almost exclusively seen in the ventral columns of the lower spinal cord. In the lateral and, less commonly, in the dorsal columns throughout the spinal cord, scattered axonal swelling was the most severe lesion. After the joint treatment with EPN and 50 ppm MBK, axonal swelling was evident but not clearly identified as paranodal. Concurrent exposure to EPN and 10 ppm MBK rarely produced large axonal swellings (Fig. 5). Swollen axons were present in dorsal, lateral, and ventral columns. Degeneration of axons was most severe in the ventral columns, but was observed in the lateral and dorsal columns of the upper cord as well.

Relative Neurotoxicity

The relative neurotoxic efficacy of EPN, MBK, or simultaneous exposure to both neurotoxic chemicals was quantified according to the neurotoxicity index (NTI) described under Methods and shown in Table 3. In this study acetone-control hens remained

TABLE 2

HISTOPATHOLOGICAL CHANGES IN NERVOUS SYSTEM TISSUES OF HENS FOLLOWING SUBCHRONIC (90 DAYS) EXPOSURE TO A DAILY DERMAL DOSE OF EPN, INHALATION OF MBK VAPORS, OR BOTH[a]

EPN (mg/kg)	MBK (ppm)	Number of hens showing histopathological changes[b]		
		Spinal cord		Peripheral nerves
		+	±	+
1.0	0	0	0	0
0	100	0	0	0
1.0	10	1	2	0
1.0	50	3	2	0
1.0	100	4	1	1

[a] Treatment schedule is outlined in Table 1.

[b] +, mild to moderate degeneration of axons and myelin; ±, equivocal changes characterized by rare swollen axons without fragmentation, phagocytosis, or loss of myelin staining.

FIG. 2. Longitudinal section of methacrylate-embedded sciatic nerve from a hen exposed simultaneously to a daily dermal dose of 1 mg/kg EPN and 100 ppm MBK for 90 days. Areas of axonal swelling with foci of Wallerian-type degeneration surrounded by phagocytic cells containing myelin debris are shown. Toluidine blue, ×400.

normal and had an NTI value of one. The NTI value for a daily dermal dose of 1.0 mg/kg EPN alone was 7.5 while that of exposure to 100 ppm MBK vapor alone was 4.2. In the case of simultaneous exposure to 1.0 mg/kg EPN and MBK, NTI value increased with increasing MBK concentration. Thus, concurrent exposure to 1.0 mg/kg EPN and 10, 50, or 100 ppm MBK resulted in NTI values of 13.6, 17.9, or 22.0.

The joint neurotoxic effect of 1.0 mg/kg EPN and 100 ppm MBK was 1.9 CNC.

Effect of pretreatments of hens with MBK on hepatic microsomal enzymes: cytochrome P-450. Cytochrome *P*-450 in hepatic microsomes from control hens was only 0.27 ± 0.06 nmol/mg protein. Daily ip injection of 5 mmol/kg of M*n*BK or MBK for 3 days produced induction of microsomal cytochrome *P*-450 at 250 ± 34 or 298 ± 16% ($\bar{x}$ ± SE) of corn oil control, respectively. These values were not significantly different from each other, but were significantly ($p < 0.01$) increased over that of the corn oil controls.

Scans of these hepatic microsomes for cytochrome *P*-450 content indicated a λ_{max} of the Soret peak of the reduced cytochrome *P*-450 CO complex in M*n*BK- and MBK-treated hens to be 450 nm.

In vitro metabolism of [^{14}C]*EPN.* Hens treated, for 3 consecutive days, with 5 mmol/kg, ip, of MBK in corn oil showed a significant increase in the hepatic metabolism of EPN to EPN oxon (303% ± 22%; $p < 0.01$) compared to hens that were only given corn oil.

DISCUSSION

Simultaneous exposure to EPN and MBK rendered hens more susceptible to neurotoxicity than exposure of hens to either chemical alone. The neurotoxic action of EPN and MBK is considered potentiation, since both

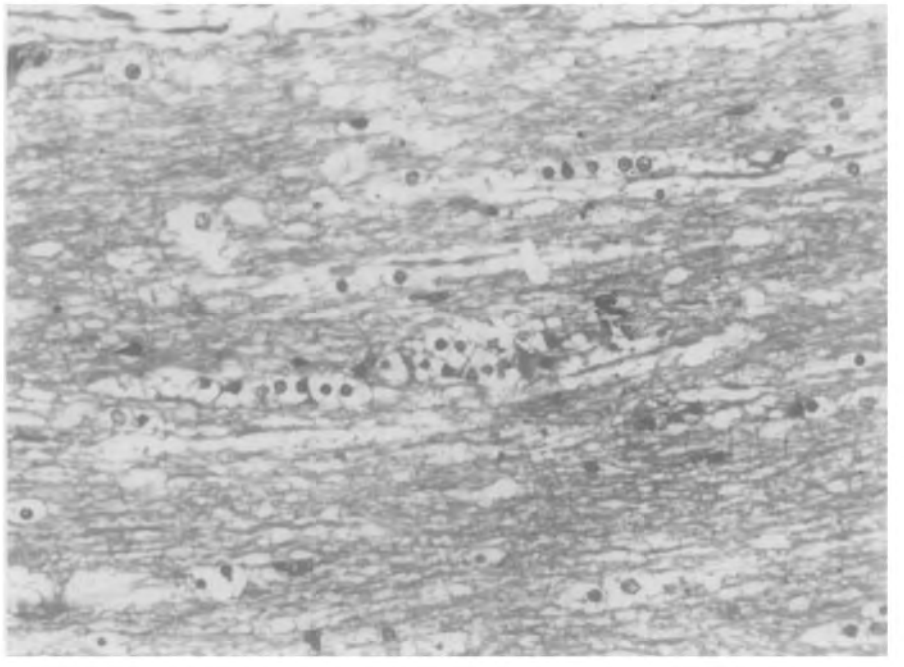

FIG. 3. Longitudinal section of methacrylate-embedded spinal cord from a hen exposed to a daily dermal dose of 1 mg/kg EPN and 100 ppm MBK for 90 days. This figure shows foci of Wallerian degeneration with associated phagocytic cells. Toluidine blue, ×400.

compounds have appreciable intrinsic neurotoxicity. In this study, hens treated with 1.0 mg/kg EPN 5 days per week for 90 days exhibited milder neurotoxic effects than when given the same treatment daily for 90 consecutive days (Abou-Donia *et al.*, 1983b). Similarly hens exposed to 100 ppm MBK for 5 days per week for 90 days were less affected when compared with those continuously exposed to MBK vapor for 90 days (Abdo *et al.*, 1982).

The signs of neurotoxicity produced in hens by either EPN or MBK were similar. Although neither chemical alone produced histopathologic lesions in this study, previous investigations have indicated that both chemicals, at higher doses, produce Wallerian-type degeneration of axons and myelin of the central and peripheral nervous systems in the chicken (Abou-Donia *et al.*, 1982, 1983b). The earlier studies have also identified characteristic differences in both distribution and morphology of the lesion produced by each chemical.

Accumulation of 10-nm neurofilaments within smaller axons is pathognomonic of neurotoxicity produced by M*n*BK and its related hexacarbon chemicals, *n*-hexane, 2,5-hexanediol, and 2,5-hexanedione which is regarded as the active neurotoxic agent of this group of chemicals. The positioning of neurofilamentous masses in the axons above the nodes of Ranvier in peripheral nerves results in the formation of giant axonal swellings (Spencer *et al.*, 1975). Similar giant axonal swellings were seen in longitudinal sections of peripheral nerves and spinal cords of hens treated with *n*-hexane and its related chemicals (Abou-Donia *et al.*, 1982; Abdo, *et al.*, 1982) and in spinal cords of similarly treated rats (Cavanagh, 1982). On the other hand, histopathologic lesions of OPIDN are characterized by destruction of neurotubules and neurofilaments first by condensation into

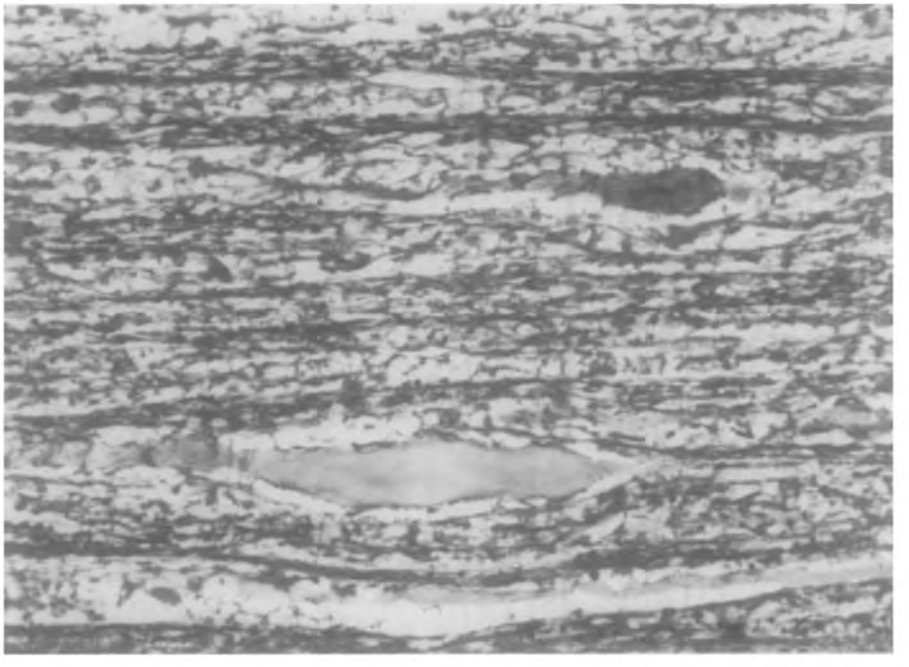

FIG. 4. Longitudinal section of paraffin-embedded spinal cord from another hen exposed to a daily dermal dose of 1 mg/kg EPN and 100 ppm MBK for 90 days. Two axons in this section of the ventral columns of the thoracic spinal cord display large paranodal swellings. No Wallerian-type degeneration is seen. H&E–LFB, ×250.

aggregates and then by conversion into more solid disordered masses (Bischoff, 1967). These changes are also accompanied by proliferation of rough endoplasmic reticulum. Recently Ca^{2+} modulation has been implicated in the development of OPIDN (Patton *et al.*, 1983; Abou-Donia *et al.*, 1984) subsequent to neurotoxic esterase phosphorylation (Johnson, 1982).

Not only are the histopathologic lesions produced by EPN and MBK morphologically distinct, but they are also distributed differently throughout the spinal cord of the hen (Abou-Donia *et al.*, 1982; Abdo *et al.*, 1982) (Fig. 6). MBK-induced axonal degeneration was exhibited almost exclusively in the ventral columns of the lumbosacral spinal cord of chickens. Infrequent swellings were the most prominent alterations seen in the lateral and, to a lesser extent, in the dorsal columns in all parts of the spinal cord.

The present results demonstrate that concurrent exposure of chickens to daily dermal doses of EPN and MBK vapor produced both types of histopathologic changes. Since only one dose of EPN was applied, the predominance of one lesion over the other was determined by the concentration of MBK vapor. Thus, high concentration of MBK produced Wallerian-type degeneration along with giant paranodal axonal swellings characteristic of MBK-induced lesions. By contrast, when hens were exposed to low MBK concentration and the same dose of EPN, only minimal histopathologic changes characteristic of EPN-induced Wallerian-type degeneration were present. After this treatment swollen axons rarely appeared.

Since the cellular mechanisms for neurotoxicity produced by EPN or MBK alone are different, the potentiating effect, reported here, could not be classified as a receptor site

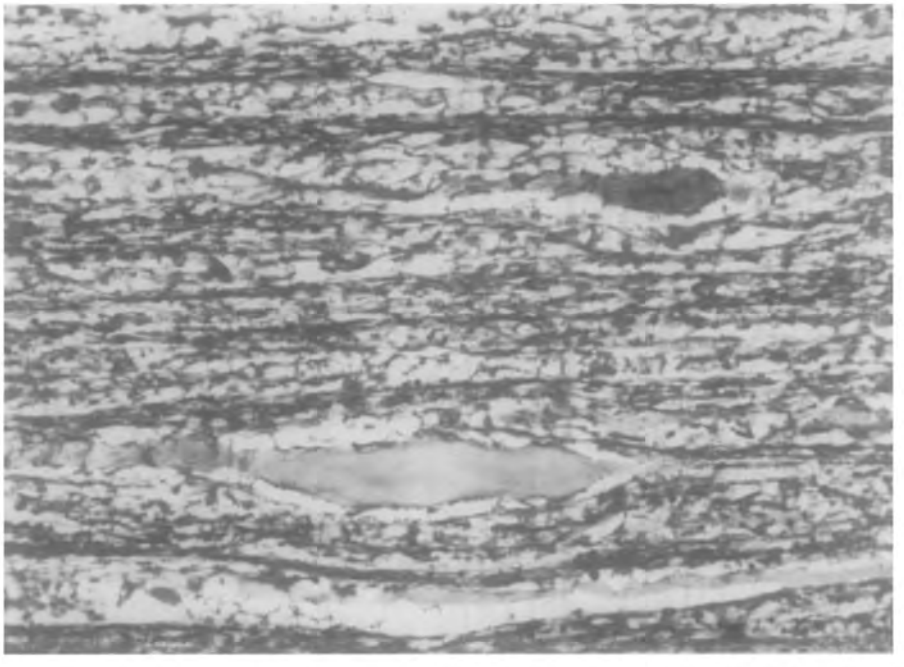

FIG. 5. Longitudinal section of paraffin-embedded spinal cord from a hen exposed to a daily dermal dose of 1 mg/kg EPN and 100 ppm MBK for 90 days. Wallerian-type degeneration is observed in this longitudinal section of the ventral columns of the thoracic spinal cord. Numerous macrophages contain myelin debris. H&E–LFB, ×250.

interaction per se. The neurotoxic actions of these chemicals were increased by experimental conditions that alter their metabolic activation or detoxification. The relative contribution of these pathways to the potentiation of EPN and MBK is unknown. The results that pure M*n*BK and technical grade MBK (M*n*BK:M*i*BK, 7:3) were of equal potency in inducing chicken hepatic microsomal cytochrome *P*-450, an enzyme involved in the metabolism of xenobiotics, are in agreement with the finding that M*n*BK and M*i*BK exhibited similar potency in inducing this enzyme (unpublished data). Furthermore, M*n*BK may also be metabolized to 5-hydroxy-2-hexanone and 2,5-HD, both of which are neurotoxic. Thus in MBK-treated hens in this study, potentiation of the neurotoxic effects of EPN could have arisen from the action of M*n*BK, 2,5-HD, 5-hydroxy-2-hexanone, and/or M*i*BK and its metabolites. It therefore seems likely that the increased rate of EPN activation, to EPN oxon, is at least partly responsible for MBK-induced potentiation of EPN nervous system injury. Previous studies demonstrated that the absorption, distribution, metabolism, and elimination of EPN follows complex pharmacokinetics (Abou-Donia *et al.*, 1983a,c). The general features of this model indicate that, for daily dermal application, absorption from the skin is rapid, and there is a rapid distribution to depots of body fat with subsequent reabsorption into the blood compartment and distribution in the nervous system. Measurable concentrations of EPN and EPN oxon have been recorded for up to 15 days after the last of the 10 applied doses.

This observation is consistent with previous reports demonstrating the potentiating ability of MBK to the hepatotoxicity of chloroform (Cornish and Adefuin, 1967; Hewitt *et al.*,

TABLE 3

NEUROTOXICITY INDEX OF VARIOUS TREATMENTS OF EPN AND/OR METHYL BUTYL KETONE IN HENS[a]

Treatment		Group ranking within ($\bar{x} \pm$ SE)[b]			
EPN (mg/kg)	MBK (ppm)	Clinical condition	Onset of clinical signs	Severity of histopathological changes	NTI[c]
1.0	0	8 ± 0.8	8 ± 0.8	6.5 ± 0	7.5 ± 0.5
0	100	3 ± 0.7	3 ± 0.7	6.5 ± 0	4.2 ± 0.5
1.0	10	13 ± 0.7	15 ± 2.5	12.8 ± 2.8	13.6 ± 0.7
1.0	50	18 ± 0.7	17 ± 0.7	18.6 ± 1.5	17.9 ± 0.5
1.0	100	23 ± 0.7	22 ± 0.7	21 ± 1.3	22 ± 0.6

[a] Treatment schedule is outlined in Table 1.

[b] Hens were assigned ranks within the following categories: clinical condition, onset of clinical signs, and severity of histopathological changes.

[c] Neurotoxicity index was calculated as the $\bar{x} \pm$ SE of the three ranks of hens for each of the three factors. Standard error type calculations were made to provide an indication of the variability among the values contributing to each mean.

1979, 1980). Also, pretreatment of rats with 2,5-HD rendered them more susceptible to hepatotoxicity produced by chloroform and carbon tetrachloride (Curtis *et al.*, 1979; Hewitt *et al.*, 1980; Branchflower and Pohl, 1982). Similarly 2,5-HD potentiated the hepatotoxicity produced by chloroform in male and female mice (Jernigan and Harbison, 1982) and in primary culture of adult rat hepatocytes (Jernigan *et al.*, 1983).

The similarity in the ability of M*n*BK and M*i*BK to potentiate chicken hepatic mi-

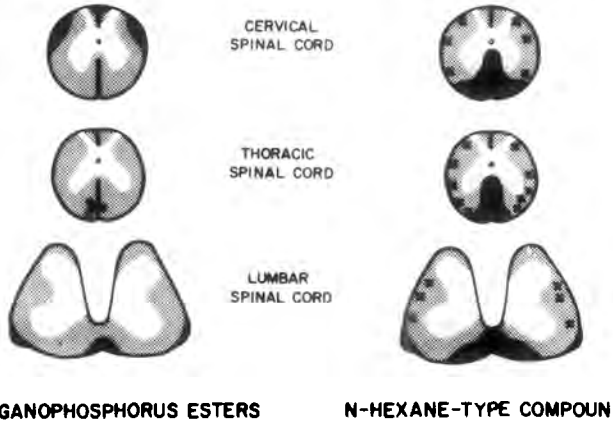

FIG. 6. Diagram showing the distribution of axonal enlargement (X) and degeneration (black areas) of the spinal cord of hens treated with neurotoxic organophosphorus compounds (e.g., EPN) or neurotoxic long-chain hexacarbons (e.g., MBK). The following tracts are damaged in OPIDN: the lateral and posterior ascending tracts in the cervical cord, early degeneration in the anterior descending tracts in the thoracic cord, and ventral columns in the lumbar cord. In long-chain hydrocarbon neurotoxicity, giant swollen and degenerated axons with phagocytosis of axons and myelin debris are present almost exclusively in the ventral columns of the lumbar cord; scattered swollen axons were seen in the lateral columns at all concentrations, while dorsal columns were the least affected.

crosomal cytochrome *P*-450 contrasts the marked disparity in their ability to produce neurotoxicity since M*i*BK is not neurotoxic (Spencer and Schaumburg, 1976). Contrary to the present results, Couri *et al.* (1977) found that continuous (7 days) exposure of rats to 750 ppm M*n*BK vapors did not alter hexobarbital sleeping time. This discrepancy may be related to species differences. However, Kramer *et al.* (1974) have shown that continuous exposure of mice for 2 to 4 days to atmospheres containing 2.5 to 3% ppm *n*-hexane induced the activity of hepatic cytochrome *P*-450. Also, MEK, a nonneurotoxic chemical, produced a synergistic effect on the neurotoxicity of M*n*BK (Abdel-Rahmen *et al.*, 1976; Saida *et al.*, 1976) and *n*-hexane (Altenkirch *et al.*, 1977). The ability of 2,5-HD to induce hepatic cytochrome *P*-450 is unknown.

Metabolic activation of M*n*BK by chicken hepatic cytochrome *P*-450 induced by EPN may also account for its potentiating action. A single po dose of 100 mg/kg EPN has been reported to produce about a 200% increase in hepatic microsomal cytochrome *P*-450 (Lasker *et al.*, 1982). It is possible that daily dermal administration of 1 mg/kg EPN for 90 days might induce cytochrome *P*-450. A consequence of this induction is the oxidative metabolism of M*n*BK to the more potent neurotoxic agent 2,5-HD.

An alternate explanation for the potentiating effect of simultaneous exposure to EPN and MBK is that the local trauma, produced by these chemicals, to the nervous tissue might, by increasing vascular permeability, increase the entry from the circulation of EPN and MBK for their active metabolites and thereby locally enhance the toxic effects, as has been suggested for 2,5-HD (Simonati *et al.*, 1983).

In summary, simultaneous treatment of hens, 5 days a week for 90 days, with dermally applied technical grade EPN and MBK vapor greatly enhanced their neurotoxic effects. This potentiation is, at least in part, a consequence of the metabolic activation and pharmacokinetic rate processes which determine the cumulative amounts of the active metabolite at the neurotoxicity target at any time after exposure. These results underline the increased hazard to humans concurrently exposed to these two types of chemicals.

ACKNOWLEDGMENTS

The authors thank Mrs. Erna S. Daniel for her secretarial assistance in the preparation of this manuscript. This study was supported by NIOSH Grant OH00823.

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